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Isolation and Characterization of Ovochymase, a Chymotrypsin-like Protease Released during *Xenopus laevis* Egg Activation

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A chymotrypsin-like protease contained in the perivitelline space of unactivated *Xenopus* eggs is released during egg activation and appears to participate in vitelline envelope conversion. This 30-kDa protease, which we have termed ovochymase, was isolated from the exudate of activated eggs using a soy bean trypsin inhibitor–agarose affinity column. The column eluant contained only two proteins, the 30-kDa ovochymase plus a 78-kDa chymotrypsin-like proteolytic activity. The 78-kDa protease was not usually observed in fresh egg exudate samples and thus was activated during the purification process and may represent the proposed precursor of the 30-kDa protease. The 30- and 78-kDa proteases were separated by gel filtration HPLC or by SDS–PAGE. The N-terminal amino acid sequence of SDS–PAGE-isolated ovochymase was determined to be VVGGQQAAPR. This conserved amino acid sequence, plus active site specific inhibition and substrate specificity studies, places ovochymase in the serine protease I family of enzymes. A two-dimensional protease activity gel revealed that ovochymase is present as several isozymes with a wide range of p*I*'s.

INTRODUCTION

As part of the block to polyspermy, activated *Xenopus laevis* eggs release proteases that are thought to be involved in conversion of the extracellular vitelline envelope to a structure which can no longer be penetrated by sperm (Grey *et al.*, 1976; Gerton and Hedrick, 1986; Lindsay and Hedrick, 1989). Characterization of proteolytic activity in the exudate of activated eggs using artificial peptide substrates, specific protease inhibitors, and SDS–PAGE showed that primarily two proteases are present, one a 45-kDa trypsin-like activity and the other a 30-kDa chymotrypsin-like activity (Lindsay and Hedrick, 1989). The use of specific protease inhibitors to block vitelline envelope conversion showed that it is the chymotrypsin-like protease which appears to be directly involved in the process, while the trypsin-like activity is required for activation of the chymotrypsin-like activity (Lindsay and Hedrick, 1989). In unactivated eggs the chymotrypsin-like protease has been localized to fibers within the perivitelline space and appears to be present in an inactive form (Lindsay *et al.*, 1992). The origin of the trypsin-like activity is unknown but is thought to be the egg cortical granules (Lindsay and Hedrick, 1989).

Although involvement of the chymotrypsin-like protease in vitelline envelope conversion has been implied through studies using specific protease inhibitors, direct evidence is lacking. Requisite to determining the exact role of this protease is its isolation. In this report we describe a simple, two-step procedure for the isolation of the 30-kDa chymotrypsin-like protease, which we have termed ovochymase. Chemical characterization of ovochymase is also presented.

MATERIALS AND METHODS

Xenopus eggs were obtained and dejellied as described previously (Lindsay and Hedrick, 1989). The eggs were then rinsed with DeBoers solution (110 m*M* NaCl, 1.3 m*M* KCl, 1.3 m*M* $CaCl_2$, to pH 7.2 with $NaHCO_3$) diluted 1/20 and placed in a dish to give a monolayer of cells covered by approximately 1 cm of buffer. The calcium ionophore A23187 (5 μM final, starting with a 5 m*M* stock solution in dimethyl sulfoxide) was then added to activate the eggs. After 30–45 min the supernatant buffer (exudate) was removed as completely as possible without damaging the eggs and then filtered through a 0.2-μm Acrodisc syringe filter (Gelman Sciences, Ann Arbor, MI). This exudate was made 10 m*M* in Tris–HCl, pH 8, by the addition of 1/100 vol of a 1 *M* stock solution followed by concentration of proteins using a stirred ultrafiltration cell (Model 8050) with a Diaflo PM10 membrane (10-kDa molecular weight cut-off, Amicon, Danvers, MA).

For isolation of the chymotrypsin-like proteolytic activity using affinity chromatography, $\frac{1}{10}$ vol of 10× loading buffer (see below) was added to the concentrated egg exudate. The sample was made 5 m*M* in benzamidine and filtered through a 5-μm Acrodisc syringe filter. This

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TABLE 1
PURIFICATION SUMMARY FOR OVOCHYMASE

	Volume (ml)	Total protein (mg)	Total activity (IU)	Recovery (%)	Specific activity (IU/mg)	Purification (x-fold)
Crude exudate	150	112	1120	100	10	1
SBTI column eluant	4	0.016	290	26	18,125	1812.5

Note. Numbers represent the average of 12 experiments.

sample was loaded onto a 1-ml column of soy bean trypsin inhibitor (SBTI)-agarose (Sigma Chemical Co., St. Louis, MO) which had been equilibrated with loading buffer (0.3 *M* NaCl, 20 m*M* Tris-HCl, 2 m*M* $CaCl_2$, pH 7.4). After loading the sample, the column was washed with loading buffer, and the ovochymase eluted with distilled water into tubes containing a drop of 1 *M* Tris-HCl, pH 8. Proteolytic activity was monitored throughout the purification procedure using the peptidyl 4-methylcoumaryl-7-amino (MCA) substrates (from Peninsula Laboratories, Belmont, CA) Suc-Ala-Ala-Pro-Phe-MCA for chymotrypsin-like activity and Boc-Phe-Ser-Arg-MCA for trypsin-like activity as described previously (Lindsay and Hedrick, 1989). In preparation for SDS-PAGE, proteins in the SBTI column eluant were precipitated by adding 1/10 vol of 100% trichloroacetic acid, plus a few drops of 10% Triton X-100 to facilitate precipitation. After 30 min on ice, the sample was centrifuged at 10,000*g* for 10 min, and the pellet was washed three times with ice-cold 90% acetone and then dried under vacuum. Sample proteins were separated on a 10% SDS-polyacrylamide gel under nonreducing conditions (Laemmli, 1970). If desired, gels were silver stained for protein (Merril *et al.*, 1981). Alternatively, using the method described by Matsudaira (1987), proteins were electroblotted onto a polyvinylidene difluoride membrane, stained using Coomassie brilliant blue, and the band of interest was excised for N-terminal amino acid sequencing. Amino acid sequence comparison was performed using the FASTA Program (Pearson and Lipman, 1988).

Protease activity gels were prepared as previously described (Lindsay and Hedrick, 1989). Briefly, samples containing active proteases (i.e., not acid precipitated) were separated on nonreducing SDS-PAGE gels containing 1% gelatin, and following electrophoresis the gels were incubated for 1 hr in 10 m*M* Tris-HCl, pH 8, containing 1% Triton X-100, then several hours in buffer without Triton X-100, and finally stained with amido black to visualize cleared areas indicating proteolytic activity. Two-dimensional gels of concentrated egg exudate were performed using a pH 3 to pH 10 ampholine range for the isoelectric focusing first dimension (O'Farrell, 1975) and a 10% nonreducing gelatin gel for the second dimension. Following electrophoresis, the gels were handled as above to visualize proteolytic activity.

Fractionation of samples by HPLC was performed us-

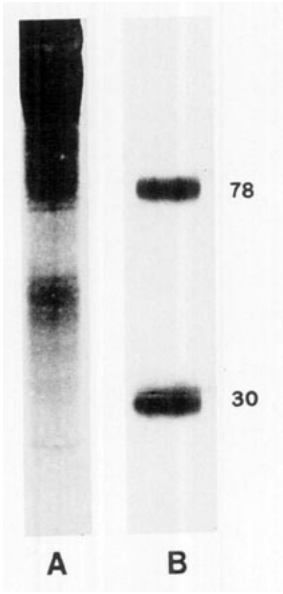

FIG. 1. A silver-stained SDS-PAGE gel of (A) crude egg exudate and (B) SBTI affinity column eluant. Numbers on the right indicate molecular weights (in kDa).

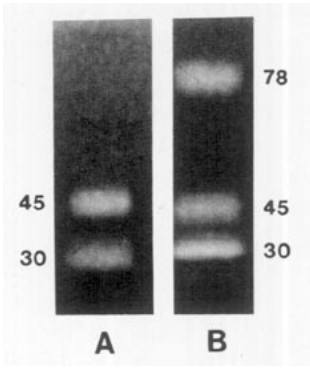

FIG. 2. Protease activity gel of (A) fresh, crude egg exudate, showing the 30 and 45 kDa activities, and (B) exudate approximately 24 hr old, showing appearance of the 78-kDa activity.

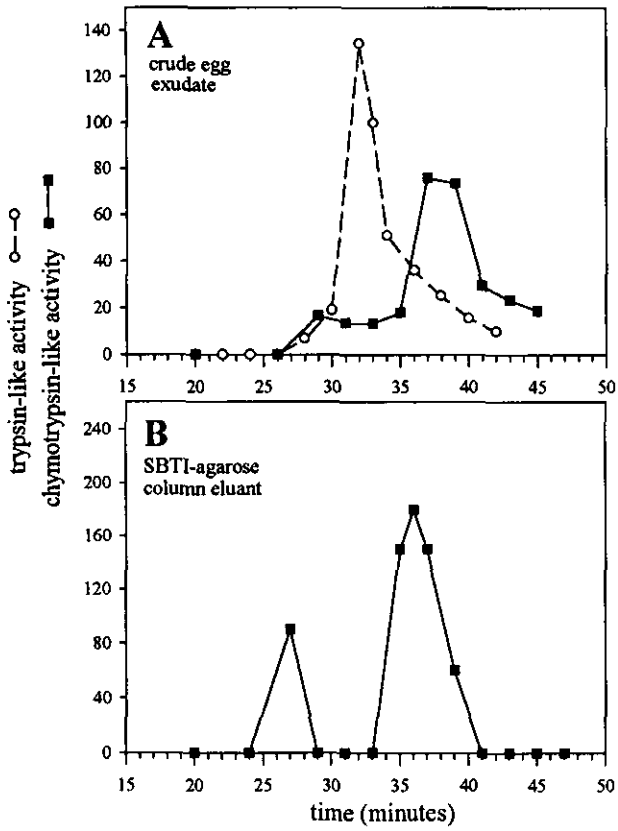

FIG. 3. Gel filtration HPLC separation of (A) crude egg exudate proteolytic activities and (B) proteases which had been eluted from the SBTI-agarose affinity column. Protease activities are presented in fluorescence units.

ing a Varian 5000 liquid chromatograph, a 50 × 1-cm TSK-3000 column (Toyosoda Limited, Japan), and a buffer of 0.1 *M* Tris acetate, 5 m*M* calcium acetate, pH 7.5, at a flow rate of 1 ml/min. Samples were concentrated and filtered, and 200 μl was loaded onto the column. Proteolytic activity in fractions was monitored using the MCA substrates as described above.

TABLE 2
N-TERMINAL AMINO ACID SEQUENCES OF *Xenopus laevis* OVOCHYMASE AND RELATED PROTEASES

Protease	N-terminal sequence
Xenopus laevis ovochymase	Val-Val-Gly-Gly-Gln-Gln-Ala-Ala-Pro-Arg-
Porcine acrosin (heavy chain)	Val-Val-Gly-Gly-Met-Ser-Ala-Glu-Pro-Gly-
Human plasmin	Val-Val-Gly-Gly-Cys-Val-Ala-His-Pro-His-
Porcine elastase	Val-Val-Gly-Gly-Thr-Glu-Ala-Gln-Arg-Asn-
Bovine trypsin	Ile-Val-Gly-Gly-Tyr-Thr-Cys-Gly-Ala-Asn-
Bovine chymotrypsin	Ile-Val-Asn-Gly-Glu-Glu-Ala-Val-Pro-Gly-
Drosophila easter	Ile-Tyr-Gly-Gly-Met-Lys-Thr-Lys-Ile-Asp-
Drosophila snake	Ile-Val-Gly-Gly-Thr-Pro-Thr-Arg-His-Gly-

Note. Sequence homology to ovochymase is indicated by underlined amino acids.

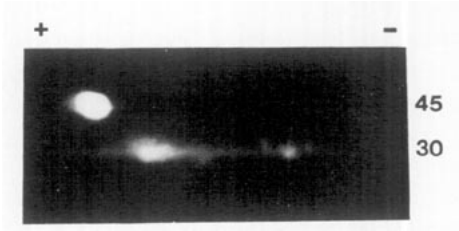

FIG. 4. A two-dimensional protease activity gel showing the 45-kDa trypsin-like protease at a single, acidic p*I*, and the 30-kDa ovochymase activity at several p*I*'s. The isoelectric focusing was run horizontally (pH 3 to pH 10), followed by SDS-PAGE separation vertically.

RESULTS

Purification of Ovochymase

Previous studies of *Xenopus* egg activation have indicated that ovochymase is converted from an inactive to an active form by the trypsin-like activity (Lindsay and Hedrick, 1989; Lindsay *et al.*, 1992). In accordance with this, we observed that ovochymase activity in egg exudate increased over the course of several hours or days, and thus, as a first step in purifying ovochymase, we allowed the exudate to sit overnight at 4°C. The advantage of this was twofold; the ovochymase activity was increased to approximately 150%, while the trypsin-like activity decreased to about 10%. We then utilized SBTI-agarose affinity chromatography to separate the proteases from other exudate components, since SBTI was determined to be a potent inhibitor of both protease activities (Lindsay and Hedrick, 1989). To focus on isolation of the ovochymase, binding of the trypsin-like protease to the SBTI column was minimized by (1) adding the trypsin-specific inhibitor benzamidine to the exudate and (2) using a column-loading buffer that was relatively high in ionic strength, which reduced the trypsin-like activity by about one-half without affecting the ovochymase activity (Lindsay and Hedrick, 1989). Ovochymase activity was eluted from the column with distilled water with up to 80% recovery. A summary of the purification is presented in Table 1. Compared to the many proteins contained in the crude egg exudate (Fig. 1A) the SBTI-agarose affinity column eluant contained only two proteins, purified over 1800-fold, the expected 30-kDa ovochymase plus another, 78-kDa, protein (Fig. 1B). The 78-kDa protein, also a protease, was not seen in fresh egg exudate when analyzed on protease activity gels (Fig. 2A), but it was activated during SBTI-agarose affinity column chromatography or in crude exudate samples that were several days old (Fig. 2B), indicating activation of the protease during the purification process or after prolonged exposure to other exudate components. This was also demonstrated when using gel filtration HPLC to separate egg exudate proteolytic activities (Fig. 3). Fractionation of crude exudate showed the presence of a small amount of chymotrypsin-like activ-

ity eluting before the 45-kDa trypsin-like activity and 30-kDa ovochymase (Fig. 3A), and protease SDS–PAGE gels of the early eluting fractions showed proteolytic activity at 78 kDa (not shown). Fractionation of SBTI-agarose column eluant (containing only 30-kDa ovochymase and the 78-kDa protein as shown above) showed an increasing amount of the higher molecular weight chymotrypsin-like activity (Fig. 3B). Together these results indicate that the 78-kDa protein is also a chymotrypsin-like protease.

Characterization of Ovochymase

The eluant from the SBTI-agarose column was electroblotted, the 30-kDa band (ovochymase) was excised, and the N-terminal amino acid sequence of the protein was determined. Table 2 shows that, as expected, the N-terminal sequence is similar to those of other serine proteases. Interestingly, ovochymase appears to be related to acrosin, a sperm protease.

Egg exudate proteases were also analyzed by two-dimensional SDS–PAGE (Fig. 4). The 45-kDa trypsin-like protease possessed an acidic p*I*, while the 30-kDa ovochymase was present as several isoforms with more basic p*I*'s.

DISCUSSION

We have presented here a simple procedure for the isolation of *Xenopus* egg 30-kDa chymotrypsin-like protease activity, designated ovochymase, from egg exudate. Interestingly, during the isolation procedure a higher molecular weight (78 kDa) chymotrypsin-like protease was activated and copurified with the ovochymase. Since ovochymase appears to be activated from a proenzyme form (Lindsay and Hedrick, 1989), the 78-kDa protein may represent the proposed ovochymase precursor molecule. Now that ovochymase has been purified, antibodies to the protein can be generated which can be used to determine whether ovochymase and the 78-kDa protein are immunologically related. The antibodies can also be used for immunolocalization of the protease in unactivated eggs to verify the results of previous experiments which used a synthetic peptide substrate to localize ovochymase to the perivitelline space (Lindsay *et al.*, 1992).

The ability to isolate active ovochymase will also allow for studies of its role in vitelline envelope conversion. Does it function directly in the limited hydrolysis of envelope components (Gerton and Hedrick, 1986; Lindsay and Hedrick, 1989) or does it have an indirect effect such as activating still another, as yet unidentified, protease? These questions, as well as the isolation and function of the trypsin-like 45-kDa protease, will be the focus of future studies. It will be interesting to see how these proteases compare to those presumed to be involved in egg envelope conversion in other species such as the mouse (Moller and Wassarman, 1989). Also, perivitelline space serine proteases have been shown to be involved in *Drosophila* dorsal–ventral specification during early embryo development (Stein and Nüsslein-Volhard, 1992), by generating the ligand for the *Toll* receptor (reviewed by St. Johnston and Nüsslein-Volhard, 1992). Two genes have been identified, *easter* and *snake*, which code for serine proteases (Table 2) that are secreted into the perivitelline space as proenzymes (DeLotto and Spierer, 1986; Chasan and Anderson, 1989). A similar pathway has been proposed for *Xenopus* embryo dorsoanterior axis formation (Thomsen and Melton, 1993) although the proteases involved have not been identified.

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